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Biological Effects of an Aqueous Extract of Cigarette Smoke Condensate in Rats. II. Effect on Liver Enzymic Activity*

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MARY

An aqueous extract of cigarette smoke condensate, 0.25 mg/ml 5% sugar solution, administered as drinking fluid to Sprague-Dawley female rats induced a slight stimulatory effect on liver arylhydroxylase activity and potentiated benzo(a)pyrene-induced liver hydroxylase and menadione reductase activity.

The observation that an aqueous extract of cigarette smoke condensate (AECSC) administered as drinking solution retards gain of body weight and accelerates induction of fibrosarcoma in Sprague-Dawley female rats after the subcutaneous injection of benzo(a)pyrene prompted us to examine the effect of this mixture on liver enzymic activity. Benzo(a)pyrene is considered to be the active (or ultimate) carcinogen by most investigators in chemical carcinogenesis. It is excreted principally via the alimentary tract irrespective of the route of administration (1,2) and the liver appears to be the major organ involved in its metabolism (3,4) although intestine, lung and epidermis (5,6,7,8) may contribute to some extent. If AECSC inhibited liver enzymic activity, its effect on B(a)P-induced sarcoma could be explained on the basis of the accepted theory of B(a)P-induced cancer. The studies presented in this report indicate that it potentiates liver enzymic activity induced by B(a)P. The component(s) of tobacco smoke condensate which cause this effect have not been identified.

RIALS AND METHODS

An aqueous extract of cigarette smoke condensate (AECSC), 25 mg/ml
illed water, was prepared each week as described in the preceding paper
stored in a light-protected polyethylene bottle at 10°. Immediately
re use each day a concentration of 0.25 mg/ml of 5% sugar solution was
ared; this mixture was used as drinking fluid for one-half of the animals
est. Benzo(a)pyrene, B(a)P, purchased from Eastman Organic Distillation
ucts, was purified on a florisil column using hexane-benzene 9:1 v:v
dried under reduced pressure. The purified product moved as a single
on a thin layer (silica gel G) chromatogram developed with benzene-
nol 19:1 v:v. A concentration of 4 mg/ml sesame oil was prepared and
ed in a sealed amber vial at room temperature for the injection experi-
s. A concentration of 20 mg/ml was prepared immediately before use in
feeding experiments.

Female rats purchased from Sprague-Dawley Farms, Inc., Madison, Wis.,
housed in stainless steel cages (4 or 5 animals per cage) in a constant-
erature (24°) animal room with an alternating light-dark cycle of 12 hr.
were fed Purina rat chow ad libitum throughout the experiment. Tap water
allowed ad libitum for 1 wk. At 30 da. of age the drinking fluid was
ged to AECSC, 0.25 mg/ml 5% sugar solution or 5% sugar solution alone.
half of each group was given AECSC; the remaining half was given the same
tity of sugar solution taken by the group given AECSC. For subcutaneous
ctions 400 μg B(a)P in 0.1 ml sesame oil or 0.1 ml sesame oil alone was
n on alternate days, starting at 31 da. of age. For the feeding experi-
s 100 mg or 20 mg B(a)P, 20 mg/ml sesame oil, was given via a No. 8
ch catheter at 51 da. of age, 3 wk. after institution of the AECSC
men.

ZYME ASSAYS

A 500-mg sample of liver was obtained from each rat killed by cervical
acture 24 hr. after treatment with B(a)P (between 9-11 a.m.). The samples
:e pooled separately for each experimental group, minced and homogenized
9 volumes ice-cold 0.25 M sucrose sol. The same homogenate served as a
ırce of enzyme for each of the following assays. N_2-fluorenylacetamide
AAF) hydroxylase activity was measured by the method of Cramer, et al. (9),
zo(a)pyrene hydroxylase activity by the method of Nebert and Gelboin (10)
menadione reductase activity by the method described by Williams-Ashman
Huggins (11). Protein was determined by the method of Lowry, et al. (12).
the hydroxylase assays crude liver homogenate equivalent to 25 mg liver
used to start the reaction in flasks containing NAD 0.25 μM, NADP 0.25 μM,
5 μM, gluc-6-PO_4 17 μM, potassium PO_4 buffer pH 7.8 100 μM, KCl 200 μM,
)P 50 μg in 0.1 ml ethanol or 50 μg 2-AAF in 0.1 ml methanol and H_2O
ml, final volume 3.0 ml. In every experiment standard and tissue-blanks
e incubated along with experimental and zero-time controls in a Dubnoff
ıbolic shaker in air for 1 hr. at 37°C. All determinations were performed
:riplicate.

Radioassay—Benzo(a)pyrene-^{3}H was prepared by acid-catalytic exchange
trifluoracetic acid, purified on a flurosil column using hexane-benzene
v, and then recrystallized in benzene. The purified product moved as
gle spot on a thin-layer (silica gel G) chromatogram developed in benzene-
ol 95:5 v:v. After determination of radioactivity it was mixed with
ed non-radioactive benzo(a)pyrene to give a final specific activity of
μC/μg. Liver homogenate equivalent to 25 mg liver was incubated with
B(a)P-^{3}H (1.0 μC) dissolved in 0.1 ml ethanol for one hour in a light-
ted Dubnoff metabolic shaker at 37°C using the same substrate concen-
ons employed for the 2-AAF and B(a)P hydroxylase assays. Enzyme activity
opped by the addition of 6 ml ice-cold acetone. This was added to the
ime samples prior to incubation. After standing overnight at -10° the
ts of each flask were transferred to centrifuge tubes and centrifuged
y to precipitate the acetone-insoluble material. The acetone-soluble
on was removed, concentrated under reduced pressure for removal of
e, extracted with ethyl acetate, and then dried over Na_2SO_4. This
on was dissolved in benzene and applied to the base line of a thin-
(0.25 mm silica gel G) chromatogram developed in benzene-ethanol (95:5),
en examined under ultraviolet light for identification of metabolites.
ged benzo(a)pyrene-^{3}H was identified and excluded from the metabolites
ere scraped from the plate and transferred to liquid scintillation
ng vials containing DPO-toluene (4 qm/L) and counted in a Packard
b liquid scintillation spectrometer. Appropriate corrections for
ing were made by the channels ratio method. The water-soluble fraction
unted directly. These assays were conducted in duplicate.

RESULTS

In general, animals injected with B(a)P had heavier livers absolutely and relatively than those injected with sesame oil, but the difference was not significant. This effect was most marked in the groups given AECSC to drink (table 1).

Enzymic activity after subcutaneous injection of B(a)P—Enzymic activity was increased two-fold in B(a)P-treated animals, text-figs. 1 (2-AAF hydroxylase), 2 (B(a)P hydroxylase), 3 (Menadione reductase). It was consistently higher (20-25%) in the groups given AECSC to drink. Fluctuations in enzymic activity appear to be due to biological variation rather than to age of the animals. Surprisingly, values for 2-AAF hydroxylase activity were slightly higher than those observed for benzo(a)pyrene hydroxylase (13). The greatest discrepancy in values was observed in the two B(a)P assays—B(a)P hydroxylase versus B(a)P-^{3}H in rats age 40 and 50 days (text figs. 2 and 4). These experiments were repeated and confirmed. Examination of the metabolic products on thin layer chromatograms revealed no qualitative differences in the metabolites formed in the six assays. 3-hydroxybenzo(a)pyrene, 6-hydroxybenzo(a)pyrene, benzo(a)pyrene-1,6-quinone and benzo(a)pyrene-3,6-quinone, together with two highly polar unidentified metabolites (probably dihydro-dihydroxy compounds (14)) were identified (1,2,4). All of these products are soluble in I N NaOH. The first 4 produce maximal fluorescence at 520-525 mμ (text fig. 5) when the spectrofluorometer (Aminco-Bowman) is set at 396 mμ but we have no information on the most polar compounds. The possibility that younger animals form a greater quantity of metabolites which would not be detected by this method of measurement should be considered.

Table 1—Mean liver and body weight of Sprague-Dawley female rats

Age	No. of Rats	Treatment†	Liver (gm)	Body (gm)
40 da	12	AECSC + sesame oil	4.26	109
	12	AECSC + B(a)P	4.53	106
	12	Sugar + sesame oil	3.99	106
	12	Sugar + B(a)P	4.28	104
50 da	8	AECSC + sesame oil	6.01	142
	8	AECSC + B(a)P	7.14	142
	8	Sugar + sesame oil	6.16	152
	8	Sugar + B(a)P	6.74	148
60 da	4	AECSC + sesame oil	6.76	170
	8	AECSC + B(a)P	7.98	169
	4	Sugar + sesame oil	6.47	179
	8	Sugar + B(a)P	7.51	174
70 da	4	AECSC + sesame oil	7.43	191
	4	AECSC + B(a)P	7.94	188
	4	Sugar + sesame oil	6.33	188
	4	Sugar + B(a)P	7.54	198
80 da	4	AECSC + sesame oil	7.18	201
	4	AECSC + B(a)P	7.70	187
	4	Sugar + sesame oil	6.43	211
	4	Sugar + B(a)P	7.62	204
90 da	4	AECSC + sesame oil	6.60	206
	4	AECSC + B(a)P	7.10	186
	4	Sugar + sesame oil	6.38	207
	4	Sugar + B(a)P	7.27	207

†B(a)P 400 μg in 0.1 ml sesame oil was injected subcutaneously on alternate days from age 31 da. to day prior to sacrifice. Aqueous extract of cigarette smoke condensate (AECSC) 0.25 mg/ml 5% sugar sol. was given as drinking fluid.

According to Gelboin and Blackburn (7) benzo(a)pyrene hydroxylase activity in liver from normal Sprague-Dawley male rats, 40-50 gm in weight, is close to 50 μμg/mg tissue. The intraperitoneal injection of 1 mg 3-methylcholanthrene increases enzymic activity to 350-400 μμg/mg. The values for 3-hydroxybenzo(a)pyrene observed in these experiments for female rats injected with sesame oil were 73-100 μg and for those injected with 400 μg B(a)P 165-316 μg/gm liver. These estimates were calculated from a standard curve using a minimum of 3 dilutions of 3-hydroxybenzo(a)pyrene† rather than the amount of fluorescence of 1 μμg, as described by Nebert and Gelboin (10).

†We are indebted to Dr. Hans Falk for the gift of 3-hydroxybenzo(a)pyrene.

Liver enzymic activity after feeding B(a)P—Although 2-AAF hydroxylase and menadione reductase activities were increased, oral administration of B(a)P in doses of 100 or 20 mg did not increase benzo(a)pyrene hydroxylase activity over that observed for rats injected with 400-μg doses on alternate days (table 2). Conney and Burns (15) have shown that a single injection of B(a)P (25 mg/Kg) increased liver enzymic activity 19-fold when B(a)P was used as a substrate. In our experiments the increase was, at best, only 3-fold. In the experiments reported by Watanabe, et al. (16) using Badger and Charles River rats no significant difference in B(a)P-hydroxylase activity was observed in livers from male and female rats, age 15 to 140 days. The low activity observed in Sprague-Dawley female rats may be due to the strain of rats. The similarity of the results of the two benzo(a)pyrene assays using entirely different methods provides convincing evidence that the methodology employed is reasonably accurate.

Table 2. Enzymic activity after feeding B(a)P*

No. Rats	Treatment	Liver hydroxylase activity, milliμM/mg protein			Menadione reductase milliμM/mg protein
		2-AAF	3-OH-B(a)P	B(a)P^{3}H	
4	AECSC + ses. oil, 1 ml	3.3	1.03	1.1	137
4	AECSC + B(a)P, 20 mg/ml	10.0	4.13	3.4	415
4	Sugar + ses. oil, 1 ml	3.1	0.79	0.9	147
4	Sugar + B(a)P, 20 mg/ml	9.4	3.55	2.9	354
5	AECSC + ses. oil, 5 ml	1.8	1.1	1.0	138
8	AECSC + B(a)P, 100 mg	8.9	3.5**	3.9**	551
8	Sugar + ses. oil, 5 ml	2.4	0.74	0.5	138
5	Sugar + B(a)P, 100 mg	7.9	2.6**	3.6**	465

* B(a)P (benzo(a)pyrene) was given to Sprague-Dawley female rats, age 51 da, via catheter 24 hr. prior to sacrifice.

** 0.5 milliμM/mg protein was present in zero-time control homogenates. Above values represent net changes.

Metabolism of B(a)P^{3}H by liver from rats given AECSC for 1 yr.—That AECSC does affect liver enzymic activity in the absence of B(a)P is shown in table 3. Five of 20 rats that had been drinking the condensate for one year and 5 of 20 given sugar solution alone were chosen at random for the two pools of liver homogenate used in this experiment. Duplicate samples were used in the assay. Homogenates of liver from animals given AECSC metabolized 25.8% of the added B(a)P^{3}H, nearly twice that observed in samples from rats given sugar solution alone. These values are higher than those observed for the younger animals, which could be the consequence of prolonged treatment with AECSC or of an unusual selection of animals for the assay. Menadione reductase activity, which so far has paralleled changes in aryl-hydroxylase activity, was increased 50% (28 μM/gm liver for the AECSC group and 18 μM/gm for the group given sugar solution). These observations clearly indicate that AECSC alone has a slight but definite influence on liver metabolism.

Table 3. Metabolism of benzo(a)pyrene-^{3}H (B(a)P-^{3}H) by liver from female rats, age 14 mo.*

Drinking Fluid		CPM	% Recovered	% Metabolized
AECSC, 0.25 mg/ml Sugar sol.	Zero-time control samples	45000	90.0	
	Incubated samples			
	Unchanged B(a)P-^{3}H	17050	34.01	
	Metabolites	12900	25.8	25.8
5% sugar sol.	Zero-time control samples	47500	92.5	
	Incubated samples			
	Unchanged B(a)P-^{3}H	23000	46.0	
	Metabolites	6900	13.8	13.8

* 50 µg B(a)P-^{3}H (S.A. 50 µC) were incubated with liver homogenates equivalent to 25 mg liver for 1 hr. at 37°.

DISCUSSION

The induction of liver enzymic activity by benzo(a)pyrene has been an established fact since 1957 (3). Potentiation of arylhydroxylase activity by an aqueous fraction of cigarette smoke condensate has not been hitherto reported. This increase is small and not statistically significant in any single assay, but the internal consistency of repeated assays indicates that the effect is not likely to be a chance occurrence. Failure to detect consistent and clear-cut changes in control animals given AECSC can be attributed to the low basal levels of enzymic activity. An increase of 20 to 30 per cent would not be so readily detected. However, the radioassay data (table 3) together with the observations on 2-AAF hydroxylase activity favor the interpretation that AECSC alone does indeed have a slight stimulatory effect.

Dontenwill, et al. (17) demonstrated a decrease in zoxazolamine-induced paralysis in male rats and hamsters exposed to cigarette smoke or injected with cigarette smoke condensate. For the rat, the decrease was 30-40% after exposure to cigarette smoke, 50% after injection of cigarette smoke condensate 40 mg/Kg, and 60-70% after injection of B(a)P 10 mg/Kg. In their extensive studies Conney and Burns (15) showed that B(a)P-induced liver enzymic activity was directly correlated with zoxazolamine-induced paralysis, and later Conney, et al. (18) and Mullen, et al. (19) demonstrated that pretreatment of rats with an intraperitoneal injection of B(a)P induced an increase in the metabolism of zoxazolamine. More recently Welch, et al. (20) reported an increase in arylhydroxylase activity in lung, placenta, intestine, and liver from pregnant rats exposed to cigarette smoke for 5 hr a day x 3 days. Liver hydroxylase activity was increased from 41 to 91 μg/gm liver/hr. Whether the effects observed by Dontenwill, et al. (17) and Welch, et al. (20) can be attributed to the components in the water-soluble fraction of cigarette smoke is uncertain; the percent change is the same order of magnitude. Our preliminary (unpublished) experiments indicate that nicotine may be responsible in part for the stimulatory effect of AECSC on liver enzymic activity but further work is necessary to establish with certainty that nicotine is responsible for all the effects observed.

In vitro studies by Benedict and Stedman indicate that whole cigarette smoke condensate, the particulate matter, or vapor phase, inhibits yeast alcohol dehydrogenase (21,22) and lactic dehydrogenase. Glucose-6-phosphate dehydrogenase activity was less affected. Benzo(a)pyrene, a known component of cigarette smoke, on the other hand, stimulates lactic dehydrogenase activity in rat liver (23). Thus it appears that either the source of enzyme or the direct addition of cigarette smoke components in vitro determines the effects observed with cigarette smoke condensates. The effect of AECSC on liver enzymic activity cannot be considered to be noxious in itself, but since it does potentiate B(a)P-induced 2-AAF activity, conceivably it would potentiate the effects of 2-AAF or other compounds which must be hydroxylated to cause cancer (24,25).

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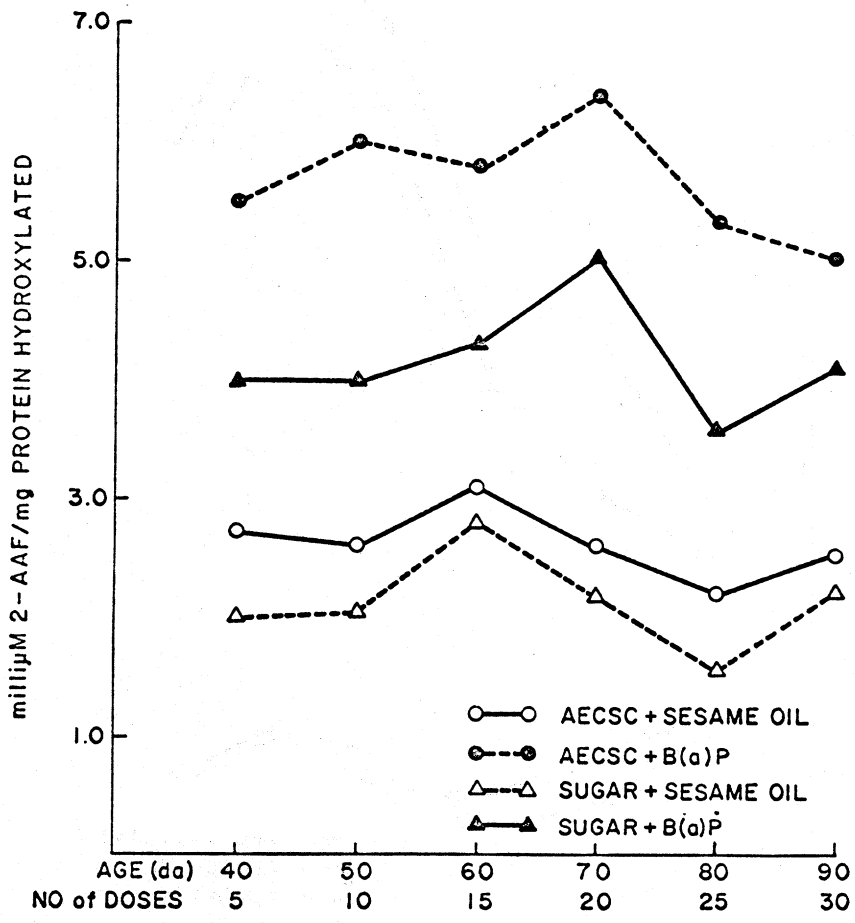
Text-figures 1-4. Influence of an aqueous extract of cigarette smoke condensate (AECSC) on liver enzymic activity. AECSC, 0.25 mg/ml 5% sugar sol., or 5% sugar sol. alone was administered to Sprague-Dawley female rats as drinking fluid from 30 da. of age until autopsy. Benzo(a)pyrene (B(a)P), 400 μg in 0.1 ml sesame oil, or 0.1 ml sesame oil was given by subcutaneous injection on alternate days. Liver enzymic activity was determined 24 hr. after injection of B(a)P or sesame oil. Text-figure 1, N_2-fluorenylacetamide (2-AAF) hydroxylase, text-figure 2, B(a)P hydroxylase measured spectrofluorometrically as 3-hydroxybenzo(a)pyrene, text-figure 3, menadione reductase, and text-figure 4, metabolism of benzo(a)-pyrene-^3H measured by recovery of radioactive metabolites.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5.0
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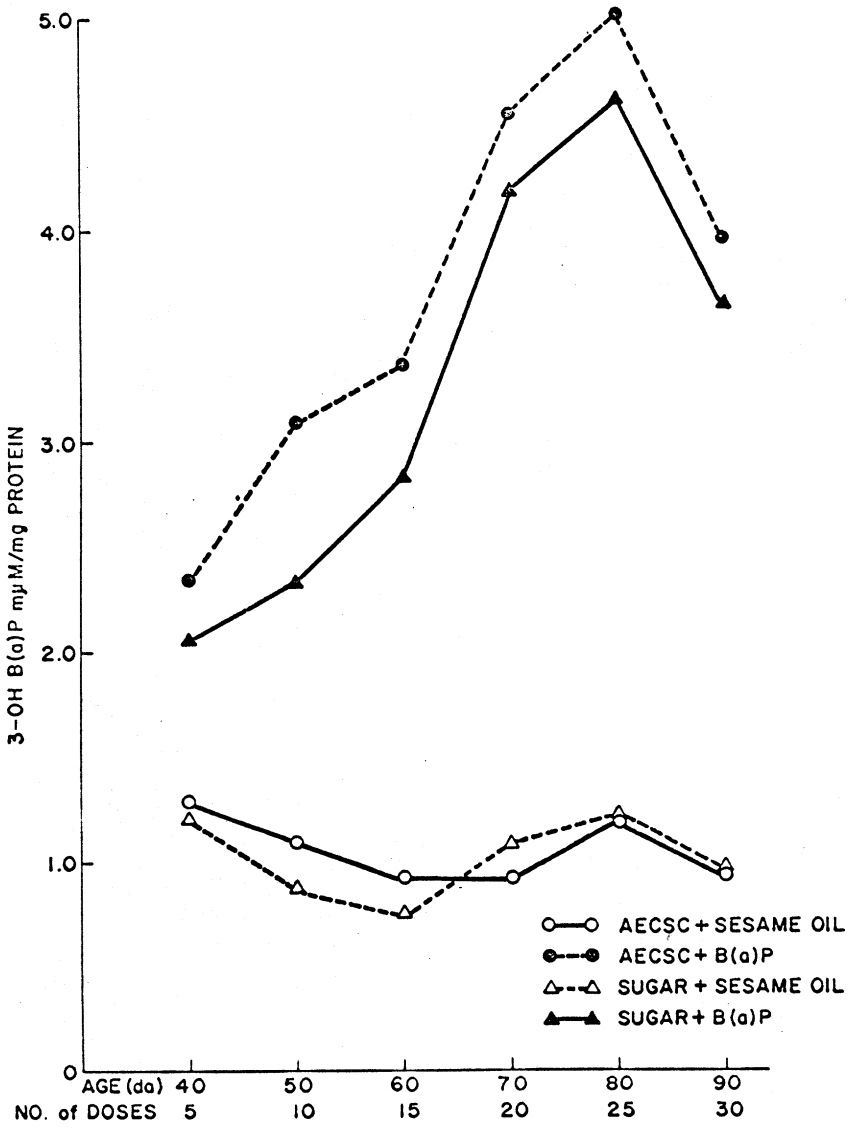
AECSC + SESAME OIL
AECSC + B(a)P
SUGAR + SESAME OIL
SUGAR + B(a)P
AGE (da) 40 50 60 70 80 90
NO of DOSES 5 10 15 20 25 30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AECSC + B(a)P
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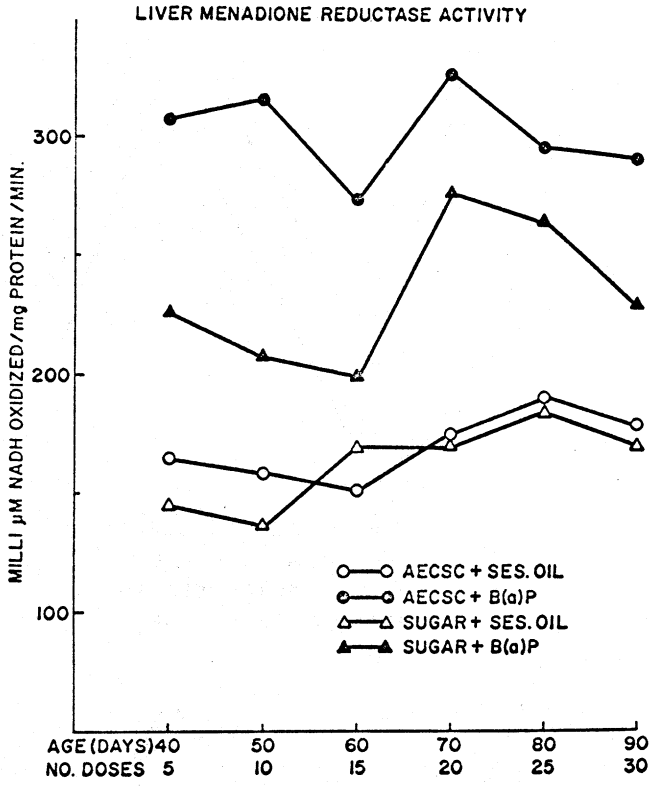
SUGAR + B(a)P


LIVER MENADIONE REDUCTASE ACTIVITY
MILLI µM NADH OXIDIZED/mg PROTEIN/MIN.
300
200
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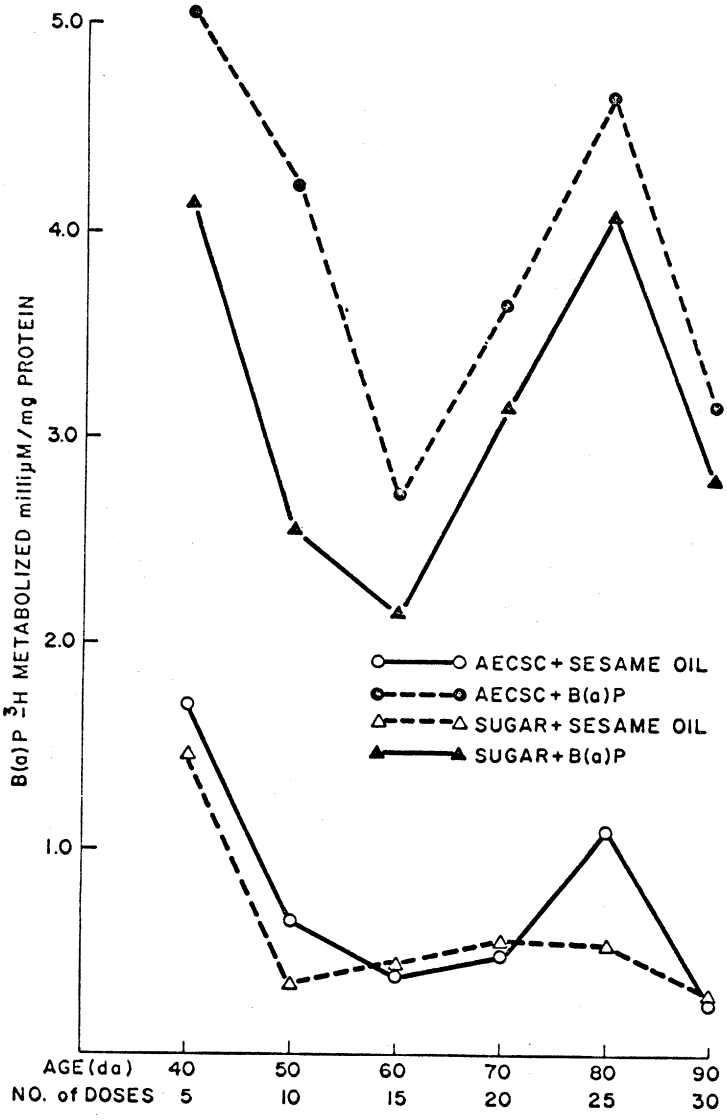
AECSC + SES. OIL
AECSC + B(a)P
SUGAR + SES. OIL
SUGAR + B(a)P
AGE (DAYS) 40 50 60 70 80 90
NO. DOSES 5 10 15 20 25 30


5.0
4.0
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B(a)P ^{3}H METABOLIZED milliμM/mg PROTEIN
AECSC + SESAME OIL
AECSC + B(a)P
SUGAR + SESAME OIL
SUGAR + B(a)P
AGE (da) 40 50 60 70 80 90
NO. of DOSES 5 10 15 20 25 30

Text-figure 5. Log-log plot (relative intensity versus concentration) of 3 known metabolites of benzo(a)pyrene in 1 N NaOH. Aminco-Bowman spectrofluorometer: activation wavelength 396 mμ; emission wavelength 522 mμ. (Benzo(a)pyrene-3,6-quinone is not shown but is soluble in NaOH). Estimates of 3-hydroxybenzo(a)pyrene formed were based on a standard curve determined in each assay using 3 or more concentrations of 3-hydroxybenzo(a)pyrene.